FTIR spectroscopy of bacteriorhodopsin microcrystals at Beamline 1.4

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INTRODUCTION

Bacteriorhodopsin (bR) is the sole protein component of the purple membrane of *Halobacterium salinarium*.[1] The function of bR *in vivo* is to convert solar energy into a pH gradient across the cell membrane which the organism uses to drive ATP synthesis.[2] Bacteriorhodopsin undergoes a light-induced cycle of physicochemical changes for for every proton it pumps out of the cell. The photocycle of bR (Figure 1) has been well-characterized by both visible and IR spectroscopy. The major intermediates are identified as the K, L, M, N and O intermediates, and each has a distinct visible color and a distinct IR spectrum.[3] The Schiff base that connects the side chain of Lys 216 to the retinal molecule buried within the core of the apoprotein[4] is deprotonated upon formation of the M intermediate, and reprotonated when the M intermediate decays. Since access to the Schiff base switches from the extracellular side of the membrane to the cytoplasmic side between these two proton transfer events, the M intermediate is of particular interest.

High resolution x-ray diffraction experiments on microcrystals of bR have recently become possible, through the discovery by Landau and Rosenbusch that the solubilized protein can be crystallized from the bicontinuous lipid-water gel that is formed by mono-olein.[5] Structural studies on intermediate states of the photocycle thus become a high priority, allowing the visualization of the structural changes that are responsible for converting light energy into a proton-motive force.

We have collected high resolution x-ray diffraction data on bR crystals kinetically trapped in both the L and M states by illumination at low temperature; refinement of the respective atomic models is close to completion. Crucial to this undertaking, however, is the confirmation by a method other than diffraction that the desired photointermediates were indeed trapped. Using Fourier Transform IR (FTIR) spectroscopy we have been able to show that we trap the bR molecules in the crystals in an unexpectedly early stage of the M state at 230K. At 170K the L state is formed, as anticipated.

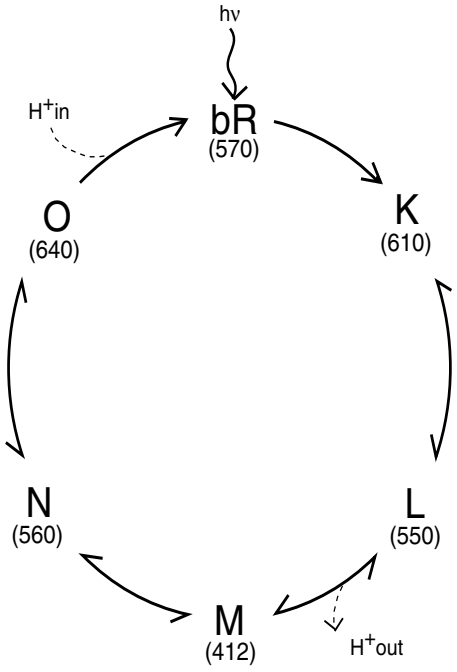

Figure 1. Key steps in the photocycle of bacteriorhodopsin. Photoexcitation of the light-adapted resting state (bR570) initiates the photocycle, represented by the five major photointermediates. The optical absorbance maximum of each is given in parentheses; photon exchange with the buldk aqueous phases on either side of the membrane is also indicated.

RESULTS

Formation of a metastable M intermediate in bR molecules in situ (i.e., in purple membrane) upon illumination with yellow light at 230K has been amply documented (reviewed in Ref. 6). Initially the FTIR spectra we collected were anomalous in that the positive difference peak at 1560 cm-1 in the average M-bR570 difference spectrum was much smaller than expected (Figure 2a). However, we subsequently obtained a second set of data, this time being careful to take spectra only from crystals that were well-

hydrated, judging by the size of the 0H stretch peak at 3400, which is diagnostic for water. The average M-bR570 difference spectrum yielded by this second set of data (Figure 2b) has a positive peak at 1560 cm-1 that is very similar to that obtained from purple membrane at 230K.[7] On the other hand, very small negative peaks in the amide I region, at 1660 and 1670 cm-1, indicate that the protein backbone has undergone very little conformational change after illumination. Based on this we conclude that a very early stage of the M intermediate is trapped in bR microcrystals at 230K, the temperature at which one of our x-ray diffraction data sets was collected.

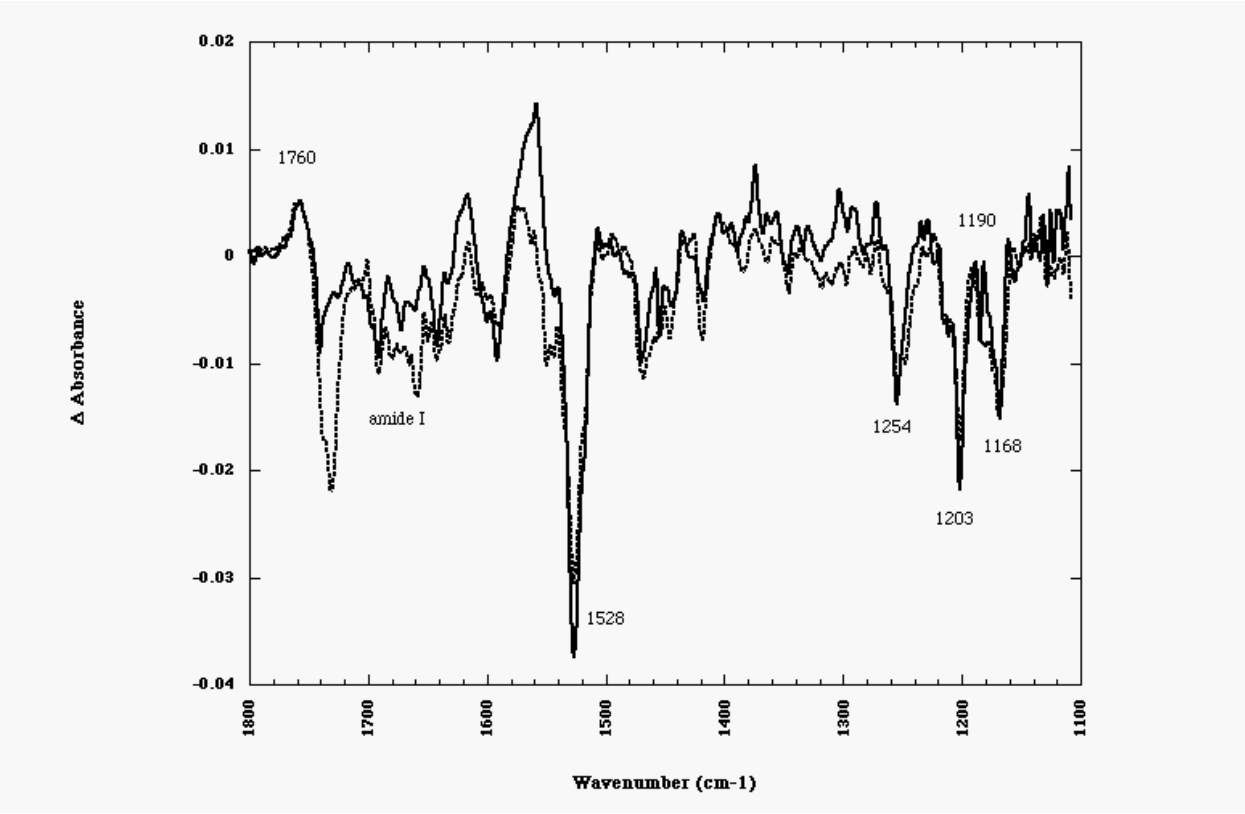

Figure 2. M-bR570 FTIR difference spectra. IR spectra at a resolution of 4 cm-1 were collected at Beamline 1.4.3 from specimens containing bacteriorhodopsin microcrystals that were cooled to 230K and either illuminated with yellow light to form the M intermediate, or not, and arithmetically averaged. The two spectra were then subtracted. Annotations identify salient features of the spectrum: 1) the positive band at ~1760 cm-1 signifies the protonation of Asp 85; 2) the negative bands at ~1660 and 1670 cm-1 are in the amide I region and indicate changes in the protein backbone conformation; 3) the negative bands at ~1528 cm-1 and between 1260 and 1160 cm-1 reflect the isomerization of the retinal group; and 4) the absence of a positive peak at ~1190 cm-1 denotes the deprotonation of the Schiff base. Features 1) and 4) are diagnostic for the M intermediate. a) The spectrum represented by the dotted line is an average of data collected without regard to the hydration state of the protein in the crystals. b) The spectrum represented by the solid line is the average of data collected more recently, after our recognition of the importance of only using well-hydrated bacteriorhodopsin crystals.

Confirmation of the trapping of the L intermediate in bR microcrystals upon illumination at 170K was obtained when we prepared an average difference spectrum from data collected at this temperature (Figure 3). Importantly, positive peaks appeared at 1410 and 1190 cm^{-1}, indicating that the Schiff base remained protonated. The short positive peak at 1560 cm^{-1} is also associated with formation of L rather than M or N,[8] but the sharp positive peaks at 1650, 1710, and 1750 are not typical of any of these intermediates. The meaning of these anomalies is unclear. Collection of more data would at least show whether these features are reproducible or not.

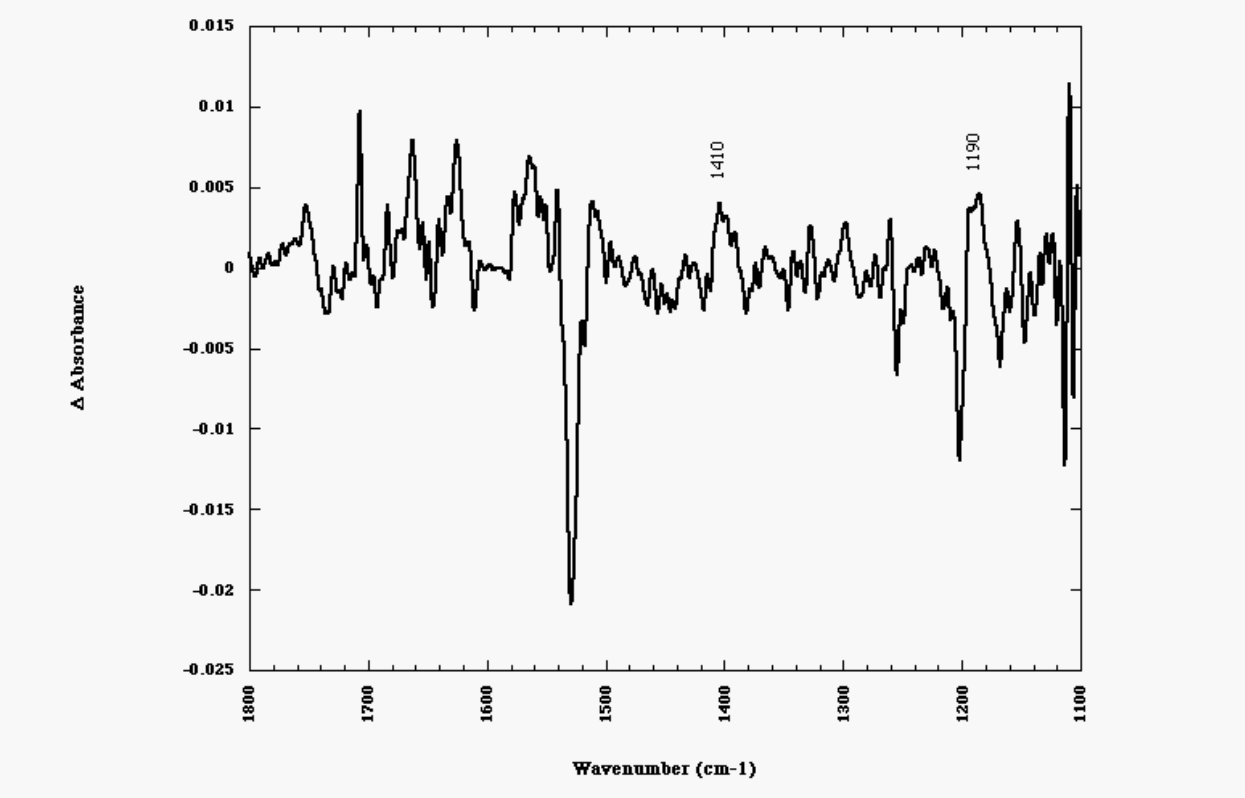

Figure 3. An L-bR570 FTIR difference spectrum was obtained as above, but with specimens set at a temperature of 170K. These data were collected from a single well-hyrdrated crystal.

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